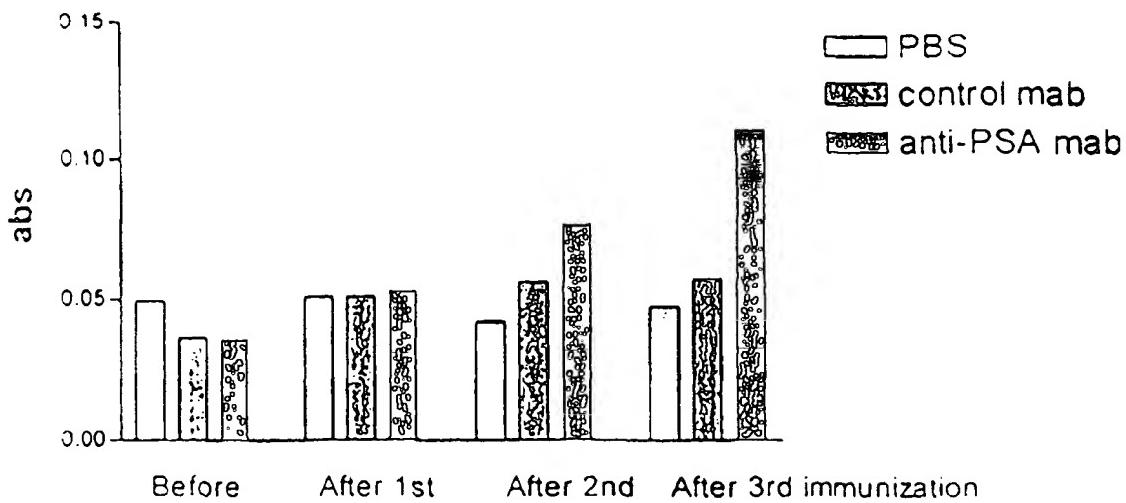




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(54) Title: IMMUNOTHERAPEUTIC COMPOSITION AND METHOD FOR THE TREATMENT OF PROSTATE CANCER



(57) Abstract

The invention is a composition and method for the treatment of prostate cancer, and involves administering an antibody that specifically binds with prostate antigen.

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IMMUNOTHERAPEUTIC COMPOSITION AND METHOD FOR THE TREATMENT OF PROSTATE CANCER

5

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to the generation of cellular and humoral immune responses to prostate-specific antigen (PSA). The present invention also relates to a therapeutic composition that contains a monoclonal antibody that is useful in generating 9 cellular and humoral immune responses to PSA.

BACKGROUND OF THE INVENTION

Prostate cancer is one of the most common cancers in men and is one of the most 13 common causes of cancer death, although, if diagnosed early, is potentially curable through surgical intervention, radiation therapy, or hormonal therapy. Since differential diagnosis is difficult at best, however, most prostate cancer is not diagnosed until later stages, typically after metastasis of the primary tumor. The primary therapy for metastatic prostate cancer is 17 either androgen-antagonistic agents or castration, but most relapse patients show prostate tumor cells that are androgen-independent. Currently, there are no effective chemotherapeutic agents that can control the growth of androgen-independent prostate tumor cells.

PSA is a 240 amino acid member of the glandular kallikrein gene family. This 33 kDa 21 single chain glycoprotein is a serine protease secreted by both normal and transformed epithelial cells of the prostate gland. PSA can be detected at low level in the sera of healthy males without evidence of prostate cancer. However, during neoplastic states, circulating 25 levels of this antigen increase markedly, correlating with the clinical stage of the disease. PSA expression is almost exclusively restricted to the prostate cells, and is the most widely used marker for the diagnosis and monitoring of prostate cancer patients.

The tissue specificity of PSA makes it a particularly attractive target antigen for the 29 development of immunotherapies against prostate cancer. PSA is detected in nearly all adenocarcinomas of the prostate and the expression of PSA was also demonstrated at distant metastatic sites. Several studies point out that PSA is a potential target for the induction of T-cell directed immunity against prostate cancer. Indeed, several PSA peptides capable of

1 binding to MHC-class I molecules have been identified and it is now well established that a
PSA specific T-cell repertoire exists in humans. Studies using in vitro immunization have
shown the generation of CD4 and CD8 cells specific for PSA and T-cell lines with specific
recognition of PSA peptides were generated from both healthy volunteer and prostate cancer
5 patients. Of particular importance is the generation in vitro of cytotoxic T-lymphocytes
specific for PSA and capable of lysing human prostate tumor cells [Xue et al., *Prostate*
30(2):73-8, (1997); Correale et al., *Journal of the National Cancer Institute* 89(4):293-300 (1997);
Correale et al., *Journal of Immunology* 161(6):3186-94 (1998); Alexander et al., *Urology* 51(1):150-7
9 (1998)].

Based on these promising data, numbers of PSA-targeted immunotherapeutic
approaches are currently being investigated in preclinical setting. Clinical trials employing a
13 recombinant vaccinia virus engineered to express PSA, recombinant PSA encapsulated into
liposomes, or autologous dendritic cells loaded with peptide sequences of PSA have already
started [See *Future Oncology* Vol 4, No. 3/4 (1998)].

The present invention describes a new immunotherapeutic product for prostate
cancer that employs as a therapeutic agent a binding agent such as a monoclonal antibody
17 directed against PSA. It is believed that the therapeutic efficacy of the injected anti-PSA
antibody is based on multiple mechanisms of actions acting in synergy. Tumor cell killing
through an ADCC or CDC mechanism is not expected for these binding agents since PSA is
not expressed at the cell surface. The therapeutic efficacy of these binding agents relies on the
21 induction of a specific PSA cellular immune response and in the non-specific stimulation of
the host immune system resulting in the induction of an immune response against various
tumor antigens.

The induction of a specific cellular immune response upon immunization of the host
25 with either Ab1 or Ab2 has been demonstrated in a number of studies. Of particular
importance is the generation through this mechanism of specific CTLs responses in ovarian
cancer patients, melanoma patients, myeloma patients, and non-Hodgkin's lymphoma
patients [Nelson et al., *Blood* 88(2):580-9 (1996); Madiyalakan et al., *Hybridoma* 16(1):41-5
29 (1997); Osterborg et al., *Blood* 91(7):2459-66 (1998); and Pride et al., *Clinical Cancer Research*
4:2363 (1998)]. It is therefore expected that the immunization of prostate cancer patients with
the binding agents of the present invention may also induce a specific and protective CTL
immune response against PSA.

1 This makes PSA an attractive target for immunotherapy. Several attempts at
generating an immune response have met with limited success [Nuc et al, *The Prostate*, 30:73-
78 (1997); Correlac et al, *J. National Cancer Institute*, 89:293-300 (1997); Choe, et al, *Cancer*
5 *Investigations*, 5:285-291 (1987); Wei, et al, *Cancer Immunol. Immunother.*, 42:362-368 (1996), and
International Application No. PCT/US97/04454, filed 19 March 1997]. Combining the
profound impact of prostate cancer with the lack of effective therapies, it is clear that
alternative modalities of treatment need to be explored and that the ability to elicit a
therapeutic immune response to PSA would be highly desirable.

9 Immunotherapies involve one or more components of the immune system to trigger a
complex cascade of biological reactions focused on eliminating a foreign molecule from the
host. The immune system consists of a wide range of distinct cell types, the most important
13 of which are the lymphocytes. Lymphocytes determine the specificity of immunity, and it is
their response that orchestrates the effector limbs of the immune system. Cells and proteins,
such as antibodies, that interact with lymphocytes play critical roles in both the presentation
of antigen and in the mediation of immunologic functions.

17 Individual lymphocytes provide a specialized function by responding to a limited set
of structurally related antigens. As noted in more detail below, this function is defined
structurally by the presence on the lymphocyte's surface membrane of receptors that are
specific for binding sites (determinants or epitopes) on the antigen. Lymphocytes differ
from each other not only in the specificity of their receptors, but also in their functions. One
21 class of lymphocytes, B cells, are precursors of antibody-secreting cells, and function as
mediators of the humoral immune response. Another class of lymphocytes, T cells, express
important regulatory functions, and are mediators of the cellular immune response.

25 Cancer immunotherapy is based on the principle of inducing the immune system to
recognize and eliminate neoplastic cells. The key elements in any immunotherapy is inducing
the host immune system to first recognize a molecule as an unwanted target, and then
inducing the system to initiate a response against that molecule. In healthy hosts, the immune
system recognizes surface features of a molecule that is not a normal constituent of the host
29 (i.e., is "foreign" to the host). Once the recognition function occurs, the host must then
direct a response against that particular foreign molecule.

Both the recognition and the response elements of the immune system involve a
highly complex cascade of biological reactions. In most immunologically based disorders, at

1 least one of the steps in the recognition phase, or at least one of the steps in the response
phase, are disrupted. Virtually any disruption in either of these complex pathways leads to
reduced response or to no response. The inability of the immune system to destroy a
5 growing tumor has been attributed, among other factors, to the presence of tumor-associated
antigens (TAAs) that induce immunological tolerance and/or immunosuppression. For
example, in some kinds of cancer, the cancer itself tricks the host into recognizing a foreign
9 cancer cell as a normal constituent, thus disrupting the recognition phase of the immune
system. The immunological approach to cancer therapy involves modification of the host-
tumor relationship so that the immune system is induced or amplifies its response to the
TAAs. If successful, inducing or amplifying the immune system can lead to tumor regression,
tumor rejection, and occasionally, to tumor cure.

13 One of the host system's mechanisms for combating a foreign molecule is called a
humoral response, the production of an antibody against a specific foreign molecule (called
an antigen). Typically, the antibody's capability of binding the antigen is based on highly
complementary structures. That is, the shape of the antibody must contain structures that are
the compliment of the structures on the antigen. When the respective structures are fully
17 complimentary, then the two molecules bind tightly.

21 Antigens are molecules that interact with specific lymphocyte receptors – surface T
cell antigen receptors and B cell immunoglobulin receptors. A particular B or T cell binds to
a very specific region of the antigen, called an antigenic determinant or epitope. Thus
antigens are molecules that bear one or more epitopes which may be recognized by specific
receptors in an immune system, a property called antigenicity.

25 Immunogenicity is the property of stimulating the immune system to generate a
specific response. Thus, all immunogens are antigens, but not vice-versa. Although an
immune system may recognize an antigen, it does not respond to the antigen unless the
antigen is also immunogenic.

29 An immune response to a particular antigen is greatly influenced by the structure and
activity of the antigen itself, as well as myriad other factors. In some cases, the immune
system is not able to generate an immune response to a particular antigen, a condition that is
called tolerance.

In influencing whether an antigen is immunogenic or immunotolerant, an important
characteristic of the antigen is the degree of difference between the antigen and similar

1 molecules within the host. The most immunogenic antigens are those that have no homologs in the host, i.e., those that are most "foreign." Other factors that promote immunogenicity include higher molecular weight, greater molecular complexity, the proper antigen dose range, the route of administration, the age of the host, and the genetic composition of the host.

5 As noted above, antigens may have one or more epitopes or binding sites that are recognized by specific receptors of the immune system. Epitopes may be formed by the primary structure of a molecule (called a sequential epitope), or may be formed by portions of the molecule separate from the primary structure that juxtapose in the secondary or tertiary
9 structure of the molecule (called a conformational epitope). Some epitopes are hidden in the three dimensional structure of the native antigen, and become immunogenic only after a conformational change in the antigen provides access to the epitope by the specific receptors of the immune system. This is an important feature and function in the ability of a
13 therapeutic reagent to initiate recognition and response to an antigen, the inducing both a cellular and humoral response to the antigen, and to increasing the antigenicity of a molecule without affecting its immunogenicity.

17 One of the responses generated by the immune system, a humoral response, involves the production of antibodies. Antibodies bear three major categories of antigen-specific determinants – isotypic, allotypic, and idiosyncratic – each of which is defined by its location on the antibody molecule. For the purpose of the present invention, we shall only focus on the idiosyncratic category.

21 Idiosyncratic determinants, or idiosyncratic markers, are markers for the V region of an antibody, a relatively large region that may include several idiosyncratic markers each capable of interacting with a different antibody. The set of idiosyncratic markers expressed on a single antibody V region constitutes the antibody idiosyncratic. An antibody (Ab₁) whose antigen combining site (paratope) interacts with an antigenic determinant on another antibody V region (idiosyncratic) is called an anti-
25 idiosyncratic antibody (Ab₂). Thus, an antibody includes an antigen binding site, and may include one or more antibody binding sites. There are two types of anti-idiosyncratic antibodies, sometimes called Ab_{2α} and Ab_{2β}. In one type of anti-idiosyncratic antibody (Ab_{2β}), the combining site perfectly mimics the structure of the antigen epitope recognized by the Ab₁ antibody. This type of anti-idiosyncratic is said to represent the internal image of the antigen. By definition, the antigen and this type of anti-idiosyncratic antibody compete for the same binding site on Ab₁, and the antigen inhibits the interaction between Ab₁ and the anti-idiosyncratic
29

1 antibody. The phenomenon of producing an anti-idiotypic antibody having the internal
image of the antigen may permit the use of antibodies to replace the antigen as an
immunogen.

5 The second type of anti-idiotype, Ab_{2α}, binds to an idiotope of Ab₁ that is distinct
from the antigen binding site, and therefore may be characterized in terms of the antigen's
inability to prevent the binding of the anti-idiotype to Ab₁. For this type of anti-idiotype,
Ab₁ can bind to both the antigen and the anti-idiotypic antibody. For a graphic
representation of these types of antibodies and their interaction, see Figure 1.

9 These various interactions based on idotypic determinants is called the idotypic
network is based on the immunogenicity of the variable regions of immunoglobulin
molecules (Ab₁) which stimulate the immune system to generate anti-idiotypic antibodies
(Ab₂), some of which mimic antigenic epitopes ("internal image") of the original antigen. The
13 presence of internal image antibodies (Ab₂) in the circulation can in turn induce the
production of anti-anti-idiotypic antibodies (Ab₃), some of which include structures that react
with the original antigen.

17 In addition to a humoral response, the immune system may also generate a cellular
response mediated by activated T-cells. There are a number of intercellular signals important
to T cell activation. Under normal circumstances an antigen degrades or is cleaved to form
antigen fragments or peptides. Presentation of antigen fragments to T-cells is the principal
function of MHC molecules, and the cells that carry out this function are called antigen-
21 presenting cells (APC: including but not limited to dendritic cells, macrophages, and B cells).

25 In addition to generating a humoral response, Ab₁ and Ab₂ have been shown to
induce a cellular immune response characterized by proliferative lymphocytes (helper and
suppressor lymphocytes), as well as cytotoxic lymphocytes. Therefore, according to the
idiotypic network theory, the injection of anti-PSA antibody should result in the induction of
a specific cellular and humoral immune response against the PSA molecule. The concept that
anti-idiotypic antibodies function as immunogens has been shown by successful
immunization against tumoral, bacterial, viral and parasitic antigens in animal models.

29 Generating Ab₂ is an indicator of the existence of a robust immune response that inherently
reflects the induction of immune system pathways.

The capture and processing of an antigen by APCs is essential for the induction of a
specific immune response. The three major APCs are dendritic cells, macrophages and B--

1 lymphocytes; dendritic cells are the most efficient. The injected antibody forms a complex with a circulating PSA, and can be targeted to dendritic cells and macrophages through the Fc-receptors present on these cells. However the high number of Fc receptors on neutrophils may considerably limit this process.

5 The capture and processing of PSA by B-cells may also occur through the interaction of the membrane bound Ab2 with the anti-PSA/PSA complexes and in a similar manner through the interaction of membrane bound Ab3 with PSA (complexed or not with the anti-PSA antibody).

9

SUMMARY OF THE INVENTION

13 The present invention is the use of a monoclonal antibody that specifically binds to a circulating prostate-specific antigen at a binding site that results in the production of anti-anti-idiotypic antibodies. A composition according to the present invention induces both a humoral and cellular immune response to PSA. Furthermore, a composition according to the present invention induces or amplifies antigenicity of a tumor-associated antigen without affecting its immunogenicity.

17 A method according to the invention preferably comprises introducing a sufficient amount of antibody into a host to stimulate a humoral and cellular immune response. The antibody composition may be formulated with an adjuvant or in a liposomal or micellular formulation.

21 The antibody according to the present invention is useful in generating a humoral and cellular immune response against PSA in a broad segment of the human population with differing HLA class I molecule types. This is in part due to the antibody's specificity for a particular sequence on the PSA molecule.

25 This sequence is of particular importance for PSA targeted immunotherapy since it contains consensus amino acid motifs for binding to HLA-A2. Of particular interest is the demonstration that the peptides 141-150, 146- 154, and 154-163 can generate in vitro human cytotoxic T-lymphocytes capable of lysing human prostate carcinoma cells. Another 29 important observation is the identification of a cleaved bond at position 145-146 of the clipped form of PSA demonstrating that this sequence is particularly sensitive to proteolytic degradation. The selected anti-PSA antibody may therefore upon binding to PSA protect this particular sequence against proteolytic degradation in the endosomal compartment of APCs

1 and enhance the production of immunogenic peptides capable to induce an anti-PSA immune response.

5 The accompanying drawings show illustrative embodiments of the invention from which these and other of the objectives, novel features and advantages will be readily apparent.

DESCRIPTION OF THE DRAWINGS

9 Figure 1 is a graphic representation of the different types of antibodies and their structural relationship to each other and to an antigen.

Figure 2 shows the amino acid sequence of a prostate-specific antigen binding site.

Figure 3 is a flow chart of the scheme for producing antibodies according to the invention.

13 Figure 4 shows the growth characteristics of an antibody according to the invention.

Figure 5 shows the binding characteristics of an antibody according to the invention.

Figure 6 shows the detection of PSA in cell supernatant of transfected cell lines.

17 Figure 7 shows that Balb/c mice (Figure 7a) and DBA mice (Figure 7b) immunized with an anti-PSA antibody of the invention, induce the production of anti-idiotype antibodies.

Figure 8 shows that immunization with an anti-PSA antibody of the invention induces the production of Ab3 antibodies.

21 Figure 9 shows the effect of immunization with an antibody of the invention on tumor development.

Figure 10 shows that immunizing with an antibody of the invention induces specific immunity against PSA. Figure 10a is for PSA and Figure 10b is for a PSA peptide.

25 DETAILED DESCRIPTION OF THE INVENTION

The present invention is a composition comprising a binding agent, such as an antibody, that specifically binds to a prostate specific antigen (PSA), preferably circulating PSA, and even more preferably to an epitope of the PSA molecule corresponding to amino acid sequences 139 to 163. In a preferred embodiment of the invention, the binding agent is a monoclonal antibody.

The present invention is a composition and method of treatment in which the composition includes a binding agent, such as an antibody, that can both induce the

1 production of an anti-anti-idiotype antibody, and can bind with circulating PSA to form a target for antigen presenting cells.

The present invention also comprises a method for treating prostate cancer by administering a composition according to the invention.

5 The present invention also comprises a composition and method for inducing the production of anti-anti-idiotypic antibodies that specifically bind to a prostate specific antigen.

The present invention also includes a kit containing a composition according to the present invention.

9 In a preferred embodiment of the invention, a composition comprising a pre-determined antibody that specifically binds to a pre-determined tumor associated antigen is used to bind a soluble antigen produced by the tumor. Once the soluble antigen is bound, the immune system recognizes the antigen as "foreign," and mounts an immune response against the antigen or against the binding agent bound to the antigen. Antigens that can be made immunogenic are potentially useful to induce or activate an immune response, leading to therapeutic and possibly prophylactic benefits.

13 As used herein the term "prostate specific antigen" includes the *in vivo*, *ex vivo*, or *in vitro* native protein, the native protein whether purified from a native source or made by recombinant technology, as well as any polypeptide, murein or portion derived therefrom that is capable of generating an immune response to a native conformationally correct PSA. For example, one can make conservative amino acid substitutions in the molecule without adversely affecting the ability to generate an antibody that will also recognize native PSA.

17 The compositions and methods of the present invention involve prostate cancer that produces a soluble multi-epitopic tumor-associated antigen (TAA). As used herein soluble is used to describe any antigen that is detectable in a body fluid, i.e., blood, serum, ascites, saliva, or the like. Prostate cancer shed soluble tumor antigens, e.g., tumor antigens shed into the bloodstream, as opposed to a surface antigen or an intracellular antigen; exhibits a multi-epitopic tumor associated antigen, preferably of protein or glycoprotein (e.g., mucin) nature; and can be found at a concentration in the patient's body fluid more than is normally present in healthy controls and such a high level signifies a poor prognosis for the patient, yet has not initiated an immune response. As is well known by one skilled in the art, one method of determining whether the concentration of the TAA is greater than is predictive of recurrence of the disease is by comparing the patient's concentration to that of a healthy control. If the

1 concentration of the TAA is higher than the healthy control, then the patient's concentration
is predictive of poor prognosis of the disease.

5 A binding agent (BA), as used herein, refers to one member of an immunologic pair,
e.g., a binding moiety that is capable of binding to a single epitope expressed on the tumor
antigen. Exemplary binding agents include, but are not limited to: monoclonal antibodies
("MAb"); chimeric monoclonal antibodies ("C-MAb"); genetically engineered monoclonal
antibodies ("G-MAb"); fragments of monoclonal antibodies (including but not limited to
"F(Ab)₂", "F(Ab)" and "Dab"); single chains representing the reactive portion of monoclonal
9 antibodies ("SC-MAb"); tumor-binding peptides; humanized antibodies; any of the above
joined to a molecule that mediates an effector function; and mimics of any of the above. The
antibody may be a polyclonal antibody or a monoclonal antibody. When the subject is a
13 human subject, the antibody may be obtained by immunizing any animal capable of mounting
a usable immune response to the antigen, such as a mouse, rat, goat sheep, rabbit or other
suitable experimental animal. In the case of a monoclonal antibody, antibody producing cells
of the immunized animal may be fused with "immortal" or "immortalized" human or
animal cells to obtain a hybridoma which produces the antibody. If desired, the genes
17 encoding one or more of the immunoglobulin chains may be cloned so that the antibody may
be produced in different host cells, and if desired, the genes may be mutated so as to alter the
sequence and hence the immunological characteristics of the antibody produced.

21 Fragments, or fragments of binding agents, may be obtained by conventional
techniques, such as by proteolytic digestion of the binding agent using pepsin, papain, or the
like; or by recombinant DNA techniques in which DNA encoding the desired fragment is
cloned and expressed in a variety of hosts. Irradiating any of the foregoing entities, e.g., by
ultraviolet light will enhance the immune response to a multi-epitopic antigen under similar
25 conditions. In a preferred embodiment of the invention, effector functions that mediate
CDC or ADCC are not required.

29 The present invention includes substitutions and deletions within the amino acid
structure of the antibody provided that the modifications result in a functionally equivalent
antibody with enhanced immunogenicity.

In an embodiment of the invention, a composition includes an antibody that
specifically binds to circulating prostate-specific antigen. In an embodiment of the invention,
the antibody binds to an epitope of PSA corresponding to amino acid sequences 139-163 of

1 the PSA molecule. More specifically, the antibody binds to an epitope corresponding to the
following amino acids: EEFLETPKKLQCVDLHVISNDVCAQV (Seq. ID No. 1). In a most
5 preferred embodiment, a binding agent according to the invention binds to an epitope of PSA
corresponding to amino acid sequences 135 to 150 of the PSA molecule. In accordance with
the present invention, other binding agents may be useful in the practice of this invention.
For example, other suitable binding agents, such as monoclonal antibodies, include those that
bind near the N-terminus of the PSA molecule.

9 The antibody of the present invention may be formulated into a pharmaceutical
composition in combination with a pharmaceutically acceptable carrier for use as an
immunogen in a mammal, preferably a human or primate. The composition may further
comprise one or more other constituents to enhance the immune response which include but
are not limited to biological response modifiers such as dendritic cell growth factors, such as
13 Flt 3; interleukin 2, interleukin 6, interleukin 12, interferon, tumor necrosis factor, GM-CSF
and cyclophosphamide. The composition may also comprise at least one HLA class I
molecule, or a cell expressing at least one HLA class I molecule in combination with one or
more antibodies or fragments thereof. Adjuvants include, for example, RIBI Detox, QS21,
17 alum and incomplete Freund's adjuvant. Liposomal formulations can also be used.

21 The composition may include one or more adjuvants, one or more carriers, one or
more excipients, one or more stabilizers, one or more imaging reagents, and/or
physiologically acceptable saline. Generally, adjuvants are substances mixed with an
immunogen in order to elicit a more marked immune response. Control vaccinations without
the adjuvant resulted in humoral immune responses. The composition may also include
25 pharmaceutically acceptable carriers. Pharmaceutically accepted carriers include but are not
limited to saline, sterile water, phosphate buffered saline, and the like. Other buffering
agents, dispersing agents, and inert non-toxic substances suitable for delivery to a patient may
be included in the compositions of the present invention. The compositions may be
solutions suitable for administration, and are typically sterile and free of undesirable
particulate matter. The compositions may be sterilized by conventional sterilization
29 techniques.

A composition according to the invention may be administered to a mammal in an
amount effective in generating a PSA specific humoral and cellular immune response. The
efficacy of the antibody as an immunogen may be determined by *in vivo* or *in vitro* parameters

1 as are known in the art. These parameters include but are not limited to antigen specific
cytotoxicity assays, regression of PSA⁺ tumors, inhibition of PSA⁺ cancer cells, production of
cytokines and the like.

5 As used herein, "administering" refers to any action that results in exposing or
contacting a binding agent, such as an antibody, with a pre-determined antigen, cell, cells, or
tissue, typically mammalian. As used herein, administering may be conducted *in vivo*, *in vitro*,
or *ex vivo*. For example, a composition may be administered by injection or through an
endoscope. Administering also includes the direct application to cells of a composition
9 according to the present invention.

13 In accordance with a method of the invention, the binding agent must be capable of
binding a pre-determined binding site or receptor, and may be administered to the patient by
any immunologically suitable route. For example, the binding agent may be introduced into
the patient by an intravenous, subcutaneous, intraperitoneal, intrathecal, intravesical,
17 intradermal, intramuscular, or intralymphatic routes. The composition may be in solution,
tablet, aerosol, or multi-phase formulation forms. Liposomes, long-circulating liposomes,
immunoliposomes, biodegradable microspheres, micelles, or the like may also be used as a
carrier, vehicle, or delivery system. Furthermore, using *ex vivo* procedures well known in the
art, blood or serum from the patient may be removed from the patient; optionally, it may be
desirable to purify the antigen in the patient's blood; the blood or serum may then be mixed
with a composition that includes a binding agent according to the invention; and the treated
21 blood or serum is returned to the patient. The clinician may compare the anti-idiotypic and
anti-isotypic responses associated with these different routes in determining the most
effective route of administration. The invention should not be limited to any particular
method of introducing the binding agent into the patient.

25 In accordance with the methods of the present invention, a composition comprising
the binding agent may be administered in an amount sufficient to recognize and bind the
prostate tumor associated antigen. In a preferred embodiment of the invention, the dosage is
sufficient to generate or elicit an immune response against the TAA. An immunologically or
29 therapeutically effective or acceptable amount of binding agent is an amount sufficient to
bind a pre-determined antigen *in vivo* or *ex vivo*, and is capable of eliciting an immune response
to the antigen. The response inhibits or kills tumor cells that carry and present a newly
accessible epitope, thereby ameliorating or eliminating the disease or condition that produces

1 the antigen. The immune response may take the form of a humoral response, a cell-mediated response, or both. In a preferred embodiment of the invention, the dosage of the monoclonal antibody is less than the dosage required to elicit ADCC or CDC.

5 The concentration or dosage of the protein in the composition can vary widely, e.g., from less than about .01% to about 15 to 20% by weight. As noted above, the composition is administered in an amount sufficient to stimulate an immune response against the antigen. Amounts effective for this use will depend in part on the severity of the disease and the status 9 of the patient's immune system. Generally, the composition will include about 0.1 µg to about 2 mg or more of protein agent per kilogram of body weight, more commonly dosages of about 1 µg to about 200 µg per kilogram of body weight. The concentration will usually be at least 0.5%; any amount may be selected primarily based on fluid volume, viscosity, antigenicity, etc., in accordance with the particular mode of administration.

13 Administration may be more than once, preferably three times over a prolonged period. As the compositions of this invention may be used for patient's in a serious disease state, i.e., life-threatening or potentially life-threatening, excesses of the binding agent may be administered if desirable. Actual methods and protocols for administering pharmaceutical 17 compositions, including dilution techniques for injections of the present compositions, are well known or will be apparent to one skilled in the art. Some of these methods and protocols are described in *Remington's Pharmaceutical Science*, Mack Publishing Co. (1982). Administration may also include *ex vivo* administration protocols, e.g., removing a portion of a 21 patient's body fluid, contacting *in vitro* the body fluid with the therapeutic composition, and then returning the treated body fluid to the patient.

25 A binding agent may be administered in combination with other binding agents, or may be administered in combination with other treatment protocols or agents, e.g., chemotherapeutic agents.

The therapeutic efficacy of the administered anti-PSA antibody is believed to depend 29 on two different mechanisms of action, although the invention is not to be restricted to the recited mechanisms. The first mechanism is the induction of a specific immunity (humoral immunity) to PSA via the idiotypic network. The second mechanism involves cellular immunity -- the administered anti-PSA antibody binds to circulating PSA to form a complex suitable for binding antigen presenting cells (APCs). Of particular interest, this mechanism of action generates a multi-epitopic immune response against the PSA. The targeting of Ar

1 47.47 at the tumor site upon i.v. injection may also favor the migration and accumulation of immunocompetent cells at the tumor site. The secretion of cytokines by these immune cells may in turn stimulate the production of a protective immune response against various tumor antigens.

5 In accordance with another embodiment of the invention, the structure of the antibody may be modified in order to enhance its capture by dendritic cells. Exemplary modifications include but are not limited to mannosylation of the anti-PSA antibody or conjugation to an antibody specifically directed against a murine dendritic cell surface marker.
9 Unlike the Fc receptors, the mannose receptor is recycled to the cell surface after endocytosis and can therefore allows internalization of ligands in successive rounds. Indeed, mannosylated proteins are presented with 100-fold higher efficiency than un-glycosylated proteins by dendritic cells.

13

EXAMPLES

Example 1. Production of AR47.47

Hybridoma clones secreting anti-PSA antibodies were produced by fusion of the murine myeloma cells Sp2/O with the splenocytes of a Balb/c mouse immunized with human PSA (figure 3).

Immunogen: PSA purified from human seminal plasma was obtained from Scripps Laboratories, San Diego, CA. [Cat # P0723 (purity, about 50%); Cat # P0725 (purity, 99%)].

21

Mice: Male Balb/cCr/AltBM mice, 6 weeks of age, were purchased from Jackson Laboratories and housed on 7th floor Dentistry/Pharmacy Building (HSLAS facilities).

Immunization protocol: Mice were immunized i.p. with 30 µg of PSA (Cat # P0723) in complete Freund adjuvant. The mice received subsequently two i.p. injections at 10 days interval with 30 µg of PSA (Cat # P0723). The first booster was performed in incomplete Freund adjuvant whereas the 2nd booster was performed in PBS. The serum levels of anti-PSA antibodies were measured by ELISA on PSA coated plates (ELISA # 1) and the mouse with the higher serum level was subjected to a final intra-splenic boost with 30 µg of PSA (Cat # P0725) in PBS.

Fusion: 3 days after the final intra-splenic boost the hybridomas were generated by fusion of spleen cells with the murine myeloma cell line Sp2/0-Ag14 (ATCC CRL-1581).

1 Screening:

First screening: Hybridoma clones secreting anti-PSA antibodies were first detected by ELISA on PSA coated plates (ELISA #1). We selected 80 positive clones from this first screening. Those clones are currently kept frozen in liquid nitrogen tank.

5 Second screening: The positive clones were further screened against PSA complexed to alpha anti-chymotrypsin (PSA-ACT) (ELISA #2); PSA non complexing to alpha anti-chymotrypsin (ELISA #4); Plasmatique kallikrein (ELISA #5); and PSA peptide 139-163 (ELISA #6)

9 The reactivity of the AR47 clones towards the different forms of PSA and towards kallikrein is reported in Example 2. The isotype of the antibodies secreted by AR47 clones is reported in Example 3.

13 The criteria of selection of a clone for additional studies was based on the reactivity towards PSA and PSA-ACT (the two main forms of immunoreactive PSA in human serum), the reactivity towards PSA peptide 139-163 (PSA sequence involved in cellular immunity), and the isotype of the secreted antibodies. Based on these criteria, the hybridoma clone AR47.47 was selected.

17

Adaptation from HAT medium to standard medium:

AR47 clones arising from 96 well plates and grown in HAT medium were first transferred into 24 well plates containing 2 ml of HT medium/well and then, once confluence was reached, transferred into 6 well plates containing 6 ml of HT medium/well. The adaptation of the cells from HT medium to standard medium was performed gradually.

HAT medium: RPMI 1640, 10% FBS, HAT supplement, OPI supplement, 0.3 mg/ml glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin

25 HT medium: RPMI 1640, 10% FBS, HT supplement, OPI supplement, 0.3 mg/ml glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin

Standard medium: RPMI 1640, 10% FBS, 0.3 mg/ml glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin.

29 Ascites can be produced in mice pre-treated with pristane (0.5 ml/mouse) and injected with AR47.47R6R6. The concentration of AR47.47 in ascites was calculated to be in the range of 4 to 6 mg/ml.

1	# of mice	# of cells injected per mouse	total # of tapping	total vol. of ascites collected
5	4	1.8×10^6	10	26 ml
	5	1.8×10^6	19	48 ml
	3	2×10^6	10	18 ml
	5	1.9×10^6	15	29 ml

Prostate specific antigen (PSA) represents an attractive target for the immunotherapy of prostate cancer. This glycoprotein is almost exclusively synthesized by the prostatic gland and is currently used for the diagnosis and monitoring of prostate cancer patients. However, since PSA is recognized as a self-antigen, it is essential for effective immunotherapy to develop innovative strategies capable of triggering the immune system and induce a protective immunity against PSA expressing cells. This example demonstrates the use of an antibody to elicit an anti-idiotype cascade associated with an antigen specific anti-tumor immune response. A large panel of anti-PSA monoclonal antibodies have been produced in our laboratory and these antibodies were evaluated for their potential therapeutic efficacy against prostate cancer. We have demonstrated that the immunization of mice with a selected anti-PSA antibody can induce a specific immunity against PSA itself. These results therefore emphasize the potential use of anti-PSA antibodies for the immunotherapy of prostate cancer.

Hybridoma clones secreting anti-PSA antibodies were produced by fusion of the murine myeloma cells Sp2/O with the splenocytes of a Balb/c mouse immunized with human PSA. An exemplary clone, AR47.47, binds to an epitope of PSA corresponding to amino acid sequences I39-163 of the PSA molecule. It has now been shown that AR 47.47 also recognizes amino acid sequences I35-I50, produces a stronger signal, and may be the minimum sequences required for binding.

The first criteria of selection used to identify the anti -PSA antibody was the ability of this antibody to interact with circulating PSA. Circulating PSA is found either in a free form or complexed to anti-proteases such as α -anti-chymotrypsin and α 2-macroglobulin. To screen for clones we used three different forms of PSA: free PSA; PSA complexed to α -anti-chymotrypsin (PSA-ACT); and free PSA non complexing to α -anti-chymotrypsin (PSA-nc). Free PSA corresponds to PSA directly purified from human seminal fluid. Co-incubating free PSA with

1 purified ACT results in the formation of PSA-ACT and PSA-nc. PSA-nc can be separated by gel
filtration chromatography. It is believed that PSA-nc may represent the free form of PSA present
in the circulation. Complexing of PSA with α 2-macroglobulin results in the total encapsulation of
PSA. As a consequence, this form of PSA is no longer detectable by monoclonal anti-PSA
5 antibodies. We therefore did not use this form of circulating PSA for the screening.

PSA belongs to the kallikrein family and a high degree of structural homology is found
between PSA and the kallikreins HK1 and HK2. The absence of cross reactivity of the anti-PSA
antibody with kallikrein isolated from human plasma was used as second criteria for selection.

9 The hybridoma clone AR47.47 responded to the criteria described above, a strong
immunoreactivity was observed with the three forms of PSA used for the screening whereas no
cross reactivity was observed with human plasmatic kallikrein. The hybridoma clone AR47.47 was
cloned twice by limiting dilution and the second generation clone AR47.47R6R6 was chosen for
13 further studies. Clone AR47.47R6R6 was adapted to standard medium (RPMI 10% FBS) and a
cell bank was formed. The absence of mycoplasma contamination was verified by using the
Boehringer Manheim mycoplasma test. Clone AR47.47R6R6 has been deposited in the American
Type Culture Collection, and has received Accession No. H-B 12526.

17 Immunization in DBA mice with a binding composition according to the invention
(AR47.47) was examined for the induction of a specific PSA immunity via the idiosyncratic network
(i.e. induction of Ab3 antibodies). Anti-PSA antibodies (Ab3) could be detected in the serum of
animals immunized with AR 47.47, a minimum of two injections of AR 47.47 was required for
21 Ab3 production. No reactivity towards PSA was detected for the control groups (mice immunized
with an isotype matched control antibody not related to PSA and mice receiving PBS injections).

25 AR 47.47 is directed towards a PSA epitope comprised between the sequence 139-163 of
the PSA molecule. The anti-PSA antibodies produced by AR 47.47 immunized mice can
specifically interact with the PSA peptide 139-163, showing that at least part of the Ab3 produced
are identical in term of specificity to AR 47.47. These results demonstrate that the immunization
with AR 47.47 can induce a specific anti-PSA immunity in the host.

29 **Example 2. Clone culture and productivity**

Growth characteristics in standard medium

Using standard medium (RPMI 1640, 10% FBS, 0.3 mg/ml glutamine, 100 μ g/ml

1 streptomycin, 100 U/ml penicillin), AR47.47R6R6 hybridoma cells were seeded at 5×10^4 cells/ml in 6 well plates and cultured for several days. Each day the cell concentration was defined by counting the cells manually with an hematocytometer, the cell viability was determined by trypan blue exclusion, and the antibody concentration in the culture media was
5 measured by ELISA (ELISA # 8). Three independent experiments were performed. The growth characteristics of the AR47.47R6R6 hybridoma clone are shown in Figure 4 and the following table:

	Exp. #1 FBS Lot #1	Exp. #2 FBS Lot #1	Exp. #3 FBS Lot #2
Maximum cell concentration (million viable cells/ml)	0.63	0.66	0.61
Maximum viability (%)	83	89	92
generation time (hr)	19.5	21.9	26.4
Maximum MAb secretion (μg/ml)	65	61.3	57.4

9
13 The AR47.47R6R6 clone has a generation time of 22 hours and can grow to a maximum of 0.6 millions cells/ml. Highest antibody level in the culture media was calculated to be approximately 60 μ g/ml.

17 AR47.47 hybridoma cells were adapted to serum free medium (SFM, Gibco) and protein free medium (UltraDOMA-PF, Biowhitaker). AR47.47R6R6 hybridoma cells were
21 adapted to serum free medium (SFM, Gibco, cat # 12045-084) and protein free media (UltraDOMA-PF, Bio Whittaker, cat #15-7-27 and 08-242F). AR47.47R6R6 was easily adapted to the protein free medium UltraDOMA. In this medium, the cells were best maintained when seeded at a concentration lower than 0.1 millions cells/ml.

25 The growth characteristics of AR47.47R6R6 in standard media (RPMI 10 % FBS), in RPMI 5% FBS, and in UltraDOMA are compared in the following table:

	RPMI 10%	RPMI 5%	UltraDOMA
Maximum viable cells per ml	0.6 million cells/ml	0.6 million cells/ml	0.3 million cells/ml
generation time	19.55 hours (Exp #1) 21.9 hours (Exp #2) 26.4 hours (Exp #3)	17 hours	18 hours (Exp #1) 27.5 hours (Exp #2)
Maximum antibody concentration	65 µg/ml on day 8	60 µg/ml on day 8	10 µg/ml (day 6, Exp #1) 18 µg/ml (day 8, Exp #2)

The adaptation of AR47.47R6R6 to serum free medium (SFM, Gibco) was in contrast considerably more difficult. The cells did not survive if diluted at a concentration lower than 1×10^5 cells/ml. The cells were best maintained at a cell concentration comprised between $2-4 \times 10^5$ cells/ml. The growth characteristics in this medium were not studied.

Example 3.

AR 47.47 was purified by affinity chromatography from either ascites or from cell culture medium. The affinity column used is the Gamma Bind Plus Sepharose from Pharmacia Biotech. The purification of the antibody was performed according to the manufacturer instruction protocol. The chromatography was monitored by measuring the absorbency at 280 nm (total protein concentration) and by testing the different fractions for the presence of anti-PSA antibody (ELISA # 1). The fractions containing purified antibody were concentrated and dialyzed against PBS. The purity of the antibody was analyzed by SDS-PAGE. The concentration of the antibody in the starting materials and in the purified fractions was determined by ELISA (ELISA # 8) and by measuring the absorbency at 280 nm (the conversion of the absorbency value to a concentration value was made using an $\epsilon = 1.46$).

AR47.47 is an IgG1 antibody and interacts specifically with free PSA as well as with PSA complexed with ACT. Synthetic linear PSA peptides were used to identify the epitope recognized by AR47.47 (figure 5). Direct binding as well as competitive experiments demonstrate that the epitope recognized by AR47.47R6R6 is comprised between the PSA sequence 139-163. AR47.47 does not recognize human plasmatic kallikrein. The isotype of AR47.47R6R6 was determined in an ELISA kit (Southern Biotechnology), and is a murine

1 IgG1,k antibody. AR47 antibodies specifically recognize PSA, PSA-ACT, and PSAn.

The domain of the PSA molecule comprised between the sequence 140-163 is of particular interest for the development of an immunotherapeutic product for prostate cancer. Indeed, the following PSA peptides; 141-150, 146-154, 154-163, bind to HLA-A2 molecules
5 and cytotoxic T lymphocytes specific for these peptides have been generated in vitro.

Using a direct binding assay with the N-biotinylated PSA peptide 139-163, we have demonstrated that AR47.47 is specifically directed against this sequence (ELISA #5). Only AR47.47 binds to the PSA peptide 139-163. The specificity of AR 47.47 for the PSA
9 sequence 139-163 was further confirmed by a competitive binding assay (ELISA # 7). The binding of AR47.47 to immobilized PSA can be inhibited in a dose dependent manner by N-biotinylated PSA peptide 139-163. In an attempt to identify more precisely the minimal sequence recognized by AR 47.47, we have studied, using the same competitive assay (ELISA
13 # 7), the inhibition of the binding of AR47.47 to PSA by the PSA peptides 141-150, 146-154, 154-163:

PSA 139-163	EEFLTPKKLQCVDLHVISNDVCAQV
PSA 141-150	FLTPKKLQCV
PSA 146-154	KLQCVDLHV
PSA 154-163	VISNDVCAQV

21 The 9 amino-acid short peptide sequences were not able to inhibit the binding of AR
47.47 to PSA. The absence of inhibition can be explained by AR 47.47 recognizing a
conformational epitope, and/or AR 47.47 recognizing a linear epitope but the peptide
sequences used are not representative of the minimal binding sequence of AR 47.47 for PSA
25 (for example AR 47.47 may recognizes the region 139-148 of the PSA molecule). It is worth
mentioning that all the peptide sequences used possess a cysteine. It is likely that the peptides
will dimerize immediately after solubilization by formation of a disulfide bridge between two
cysteine residues. The dimerized peptides may not be recognized by AR47.47. It has been
29 shown, however, that various antibodies recognize an epitope near the N-terminus of the
PSA molecule, and these antibodies induce a therapeutically beneficial immune response.

The measurement of the affinity of AR 47.47 for PSA was determined using either

1 radiolabeled AR 47.47 (RIA # 1) either radiolabeled PSA (RIA # 2). The Kd values
 calculated from independent experiments are reported in the following table:

Radiolabeled ligand	Specific activity ($\mu\text{Ci}/\mu\text{g}$)	affinity for PSA Kd (nM)	affinity for PSA-ACT Kd (nM)	affinity for PSA peptide Kd (nM)
AR 47.47	47 $\mu\text{Ci}/\mu\text{g}$	2.1 2	1.6 1	
AR 47.47	15.8 $\mu\text{Ci}/\mu\text{g}$	3.3		3.4
AR 47.47	29 $\mu\text{Ci}/\mu\text{g}$	2.77 2.2		2.3 1.6
PSA	7.65 $\mu\text{Ci}/\mu\text{g}$	1.1		
PSA	7.65 $\mu\text{Ci}/\mu\text{g}$	0.6		

13 The PSA sequence recognized by AR 47.47 does not contain tyrosine and therefore is likely not to be affected by the iodination process. The slightly higher affinity of AR 47.47 calculated when radiolabeled PSA is used in comparison of radiolabeled AR 47.47 may suggest that the affinity of the antibody is slightly diminished by the iodination process.

17 The affinity of AR 47.47 for PSA-ACT was performed using radiolabeled AR 47.47 (RIA #3). Two independent experiments were performed. The affinity constants calculated from the Scatchard plots were 1.6 nM and 1 nM respectively. The affinity of AR47.47 for both PSA and PSA-ACT therefore appears to be close.

21 The affinity of AR 47.47 for PSA peptide 139-166 was performed using radiolabeled AR 47.47 (RIA #4). Three independent experiments were performed. The affinity constants calculated from the Scatchard plots demonstrate similar affinity of AR 47.47 for both PSA and PSA peptide 139-163.

Source	MAb concentration in the starting materials	Total amount of antibody purified	Yield
ascites (9.5 ml)	4 mg/ml	31 mg	80%
ascites (56 ml)	4.4 mg/ml	145 mg	60%
culture medium (120 ml)	14.6 mg/ml	1.4 mg	80%

1 The fragmentation of AR47.47R6R6 antibody into F(ab)2 fragments was realized by
enzymatic digestion using immobilized ficin (Pierce) as described by the manufacturer
instructions. The separation of the Fab fragments from the Fc fragments was performed by
affinity chromatography on Protein A column. The non-bound fractions washed out of the
5 column contain the fab fragments whereas the bound fractions contained the Fc fragment
and eventually the remaining non-digested antibody.

9 The fractions eluted from the Protein A column were first tested for their
immunoreactivities with anti-Fc and anti-Fab antibodies (ELISA # 9 and 10). The results
obtained indicate that the first fractions directly eluted from the column contain as expected
Fab [either Fab or F(ab)2] fragments and were not contaminated with Fc fragments. We
further demonstrated that these Fab fragments were still able to bind PSA (ELISA # 11, RIA
#5). SDS PAGE analysis of AR 47.47 purified from GammaBind + column.

13

Example 4. Animal model

17 Two murine PSA-transfected cell lines (Line-1-PSA; P815-PSA) were obtained from
Dr. E.M. Lord (University of Rochester, NY). Line-1 is a Balb/cByJ (H-2^d) mouse lung
carcinoma cell line and P815 is a DBA/2 (H-2^d) mastocytoma cell line. The transfected cell
lines Line-1-PSA and P815-PSA are stably transfected and secrete high level of PSA in the
culture medium (Figure 6). The levels of PSA expression by these transfected cell lines is
comparable to what has been observed in the dihydrotestosterone -induced human prostate
21 cell line LnCAP [Wei et al; *Cancer Immunol Immunother*, 42:362-368 (1996)].

25 The expression of MHC class I and MHC class II molecules was studied by FACS
analysis using the commercially available antibodies directed against H-2KdDd (MHC class I)
and I-Ad (MHC class II). P815-PSA cells grown in vitro or isolated from solid tumor (from
DBA mouse) express high level of MHC class I molecules but only low levels of MHC class
II molecules. When grown in vitro, Line 1-PSA expressed very low levels of MHC class I and
II molecules. However, the expression of MHC class I and II molecules could be induced by
treatment of the cells for 5 days with 3% DMSO.

29

	MHC Class I		MHC Class II	
	negative control (mean)	MAb anti-H2K ^d d ^d (mean)	negative control (mean)	MAb anti-I-A ^d (mean)
P815-PSA				
grown in vitro	3	652	4	41
isolated from solid tumor	3	849	3	69
Line 1-PSA				
grown in vitro	4	18	15	46
isolated from solid tumor	5	91	18	113

Two syngeneic tumor models were established. The first animal model consists of Balb/cByJ mice injected intravenously with 0.1 million Line-1 -PSA tumor cells. After 3-4 weeks the mice were sacrificed and the tumor burden was calculated by measuring the number of tumor foci in the lung and by measuring the weight of the lungs.

The second animal model consists of DBA/2 mice injected subcutaneously with 0.5 million P815-PSA cells. After 2 weeks the tumors were palpable and measured at regular intervals with a caliper. Four weeks after the inoculation of the tumor cells, the mice were sacrificed, the tumors are dissected and weighed.

Example 5. Anti-idiotypic induction of PSA immunity in mice

Mice were used to determine whether immunization with anti-PSA antibodies can induce a specific immunity against PSA via activation of the idiotypic network. The goal of this experiment was to demonstrate that the immunization of mice with anti-PSA antibodies (Ab1) can stimulate the immune system to generate anti-idiotypic antibodies (Ab2 ≈surrogate antigen), and anti-anti-idiotypic antibodies (Ab3) capable of reacting with the original antigen.

These experiments used a commercially available antibody as a model anti-PSA antibody (RLSD09; ATCC HB-8525). The purified antibody was conjugated to Keyhole Limper Hemocyanin (KLH) to enhance its immunogenicity. The anti-PSA antibodies conjugated to KLH were still capable of binding to PSA, indicating that the idiotype of the antibodies were not masked by the conjugation procedure. B43.13 antibody, a mouse monoclonal antibody of the same isotype

1 as the PSA antibody (IgG1) was used as the control. B43.I3 antibody is specifically directed against
the CA125 ovarian tumor antigen and does not cross react with PSA. In addition FACS analysis
verified that the B43.I3 antibody does not bind at the cell surface of Line-1-PSA or P81 5-PSA.

5 Mice were subdivided into three groups of five mice each. The first group of mice was
immunized with anti-PSA antibody conjugated to KLH. The second group of mice was immunized
with the control B43.I3 antibody conjugated to KLH. The third group of mice received PBS
injection. Injections were performed i.p. at 10 days intervals with complete Freund adjuvant for the
first injection and incomplete Freund adjuvant for the second injection.

9 Ab2 is a surrogate antigen capable of mimicking the PSA epitope recognized by the
13 injected anti-PSA antibody. A competitive inhibition assay was established to measure the serum
level of Ab2. This assay was performed 5 days after the second injection. An inhibition was
observed after incubation in the presence of mouse sera from mice immunized with anti-PSA
antibody, but not when sera from mice immunized with control antibody or PBS were used. These
results indicate that the immunization of Balb/c mice (Figure 7A) and DBA mice (Figure 7B) with
the anti-PSA antibody can induce the formation of anti-idiotypic antibody (Ab2) capable of
mimicking PSA.

17

Example 6.

21 Ab3 corresponds to the anti-PSA antibodies produced by the host. The Ab3 level in
the mouse serum was measured by ELISA on PSA coated plate, performed 5 days after the
25 second injection (Figure 8). The anti-PSA antibodies measured by this assay can not
correspond to the injected antibodies. Anti-PSA antibodies could be detected in the mouse
serum of DBA mice and Balb/c mice immunized with anti-PSA antibody. These results
demonstrate that an anti-PSA immune response can be induced in mice by immunization
with an anti-PSA monoclonal antibody.

Example 7. Effect of Anti-PSA immunization on tumor development

29 Balb/c mice were used to determine whether immunization with anti-PSA antibodies
can protect the animals against a subsequent tumor challenge. Balb/c mice were divided into
3 groups of 5 mice each. The first group was immunized with anti-PSA antibody RLSD09
conjugated to KLH, the second group was immunized with control antibody B43 conjugated
with KLH, the third group received PBS injections. A total of 4 injections were given for each

1 group using 50 µg of antibodies for each injection. The tumor cells Line-1-PSA were injected intravenously between the third and fourth injections. Nineteen days after tumor inoculation, the mice were sacrificed, the number of tumor foci in the lungs and Ab3 levels in the serum were determined.

5 The tumor burden in the group of mice immunized with anti-PSA MAb was considerably lower compared to the group of mice immunized with control antibody. Of particular interest is the demonstration, in the group of mice immunized with anti-PSA MAb, of a negative correlation between Ab3 levels and the number of tumor foci in the lungs
9 (Figure 9).

Example 8. Effect of immunization on the induction of a specific PSA immunity

13 We have studied in DBA mice whether the immunization with a monoclonal antibody that specifically binds to PSA (AR47.47) can result in the induction of a specific PSA immunity via the idiotypic network (i.e. induction of Ab3 antibodies). Anti-PSA antibodies (Ab3) could be detected in the serum of animals immunized with AR 47.47, a minimum of
17 two injections of AR 47.47 was required for Ab3 production. No reactivity towards PSA was detected for the control groups (mice immunized with an isotype matched control antibody not related to PSA and mice receiving PBS injections). See Figure 10a.

21 Ar 47.47 is directed toward a PSA epitope comprised between the sequence 139-163 of the PSA molecule. The anti-PSA antibodies produced by AR 47.47 immunized mice can specifically interact with the PSA peptide 139-163 (Figure 10b). We can therefore conclude that at least part of the Ab3 produced are identical in terms of specificity to Ar 47.47. These results demonstrate that the immunization with AR 47.47 can induce a specific anti-PSA
25 immunity in the host.

Example 9.

The syngeneic tumor animals models used for these studies are:

- 29 A. Balb/cByJ mice injected intravenously with 0.1 - 0.04 million Line-1-PSA tumor cells. After 3-4 weeks the mice are sacrificed and the tumor burden is calculated by measuring the number of tumor foci in the lung and by measuring the weight of the lungs.
33 B. DBA/2 mice injected subcutaneously with 0.4 million P815-PSA cells. After 2 weeks

1 the tumors are palpable and measured at regular intervals with a caliper.

Approximately four weeks after the inoculation of the tumor cells, the mice are
sacrificed, the tumors are dissected and weighed.

5 Therapeutic antibodies and control antibodies

Therapeutic antibodies: AR47.47R6R6 a murine monoclonal antibodies of the IgG1 isotype specifically directed against PSA

Control antibodies: murine monoclonal antibodies of the IgG1 isotype:

9 CA 125 MAb directed against the tumor antigen CA125 (AltaRex Corp.)

MOPC-21 a mouse myeloma immunoglobulin of unknown specificity (Sigma)

The absence of interaction between control antibodies and PSA was verified by ELISA.

13 The binding of the anti-PSA antibodies and control antibodies to the PSA-transfected tumor cell lines was studied by FACS analysis. None of the anti- PSA antibodies studied bound to the surface of PSA transfected tumor cells grown in vitro. The control antibodies did not bind to the surface of Line-1-PSA tumor cells. A very low binding of the two control antibodies was observed for P815-PSA cell line.

	AR 47.47		CA 125 MAb		MOPC	
	negative control (mean)	AR 47.47 MAb (mean)	negative control (mean)	CA 125 MAb (mean)	negative control (mean)	MOPC MAb (mean)
P815-PSA	5 3	8 4	5 5	12 40	5 3	37 11
Line 1-PSA	7 7 4	7 7 4	7 7	7 7	7 7 4	8 8 5

25 Anti-PSA antibodies and control antibodies were conjugated to KLH in order to enhance their immunogenicity in mice (glutaraldehyde conjugation, 1 mole IgG / 0.2 mole KLH). The PSA binding activity of AR 47.47 after KLH-conjugation was considerably reduced by the conjugation process (90%).

	Experiment #	5	6	7	8	9
1	mice	Balb/c	DBA	Balb/c	DBA	Balb/c
	# mice/group	10	10	5	5	5
5	Therapeutic anti-PSA MAb	47.47R6R 6	47.47R6R 6	47.47R6R 6	47.47R6R 6	47.47R6R 6
	Control MAb	MOPC	MOPC	CA125 MAb	CA125 MAb	MOPC
9	form of injected MAb	KLH conjugate	KLH conjugate	KLH conjugate	KLH conjugate	KLH conjugate
	Dose of MAb per mouse/injection	50 µg				
	Tumor cells	Line 1- PSA	P815-PSA	Line 1- PSA	Line 1- PSA	P815-PSA
	tumor inoculation	i.v.	s.c.	i.v.	i.v.	s.c.

	Experiment #	10	11	12	13	14
13	mice	DBA	Balb/c	DBA	Balb/c	DBA
	# mice/group	5	5	5	5	5
17	Therapeutic anti-PSA MAb	47.47R6R 6	47.47R6R 6	47.47R6R 6	47.47R6R 6	47.47R6R 6
	Control MAb	MOPC	CA125 MAb	CA125 MAb	CA125 MAb	CA125 MAb
21	form of injected MAb	KLH conjugate	KLH conjugate	KLH conjugate	KLH conjugate	KLH conjugate
	Dose of MAb per mouse/injection	50 µg				
	Tumor cells	P815-PSA	Line 1- PSA	P815-PSA	Line 1- PSA	P815-PSA
	tumor inoculation	s.c.	i.v.	s.c.	i.v.	s.c.

1 **Example 10. INDUCTION OF A SPECIFIC ANTI-PSA IMMUNE RESPONSE IN
MICE BY IMMUNIZATION WITH AR 47.47.**

The elicitation of a specific PSA immune response upon immunization with AR 47.47 and induction of a specific idiotypic network was studied both in Balb/c mice and DBA mice. 5 In this set of experiments (experiments # 5, 6, 7, 9, 11, 12), the mice were first treated with either AR 47.47, control MAb or PBS, and then inoculated with PSA-transfected tumor cells. The results correspond to the measurement of the anti-idiotypic (Ab2) and anti-anti-idiotypic (Ab3) immune response against AR 47.47 before tumor inoculation.

9 The detection of anti-idiotypic antibodies (Ab2) was performed by ELISA using plates coated with AR 47.47 F(ab) fragments (produced by ficin digestion). The presence of Ab2 in the mouse sera of AR 47.47 immunized mice was demonstrated in experiments 7, 9, 11 and 12.

13 Two different ELISA assays were performed to detect the presence of Ab3. The first assay detects the binding of Ab3 on PSA coated plate. Using this assay, we have demonstrated in experiments # 5, 6 and 7 the presence of Ab3 in the sera of mice immunized with AR 47.47. The second assay employs the PSA peptide known to be recognized by AR 17 47.47. This assay gave positive signal for the mice immunized with AR 47.47 in experiment 7. This second assay however has not been standardized at this time and the results shown in figure 7 should be analyzed with caution since in many cases the positive control (performed with AR 47.47) showed negative signal. Since the PSA peptide used for this assay contains 21 cysteine residues we believed that a cyclisation or polymerization of the peptide occurs after solubilization and/or storage of the peptide. Such effect may impairs the binding of the peptide to streptavidin coated plate or to specific antibodies (i.e. AR 47.47 or Ab3).

25 A competitive ELISA assay employing the PSA peptide recognized by AR 47.47 was also performed. The inhibition of the binding of PSA peptide to AR 47.47 immobilized on ELISA plate is theoretically dependent on the presence of both Ab2 and Ab3 in the mouse sera. A competitive inhibition was observed for experiments # 7, 9, 11 and 12.

29 These results demonstrate that an anti-idiotypic network resulting in the formation of anti-PSA antibodies by the host could be induced in mice (Balb/c and DBA) upon immunization with AR 47.47.

1 **Example 11. EFFECT OF AR 47.47 ON TUMOR DEVELOPMENT**

The ability of AR 47.47 to inhibit the development of PSA-transfected tumors was studied in both Balb/c mice (experiment # 5, 7, 11) and DBA mice (experiment 6, 9, 12). In this set of experiments the tumor inoculation occurs after treatment of the mice with either AR 47.47, control MAb or PBS. The immunization of the mice was continued after tumor inoculation.

For each experiment, the following parameters were measured: Ab2; Ab3; Ab2 + Ab3; tumor burden. The results obtained for each experiment are shown in the following tables:

Effect of AR47.47-KLH immunization on Line 1-PSA tumor development in Balb/c mice

experiment #	# of lung tumor foci (mean ± SE)		
	PBS	control MAb-KLH	AR47.47-KLH
5	12.3 ± 3.2	8.3 ± 1.5	32.3 ± 2.2
7	26.6 ± 3.9	9.0 ± 1.5	9.6 ± 1.9
11	15.2 ± 1.2	14.8 ± 2.5	25.8 ± 7.5

Experiment t #	Ab2					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
5	-	-	-	-	-	+
7	-	-	+	-	-	+
11	-	-	+	-	-	+

Experiment t #	Ab3					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
5	-	-	+	+	+	+
7	-	-	+	+	+	+
11	-	-	-	+	+	+

Experiment t #	Ab3 peptide					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
5	-	-	±	+	+	+
7	-	-	+	-	-	+
11	-	-	-	-	-	-

Experiment t #	Ab2 + Ab3					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
5	-	-	-	-	-	+
7	-	-	+	-	-	+
11	-	-	+	-	-	+

1 Effect of AR47.47-KLH immunization on P815-PSA tumor development in DBA mice

experiment #	% of mice without tumor		
	PBS	control MAb-KLH	AR47.47-KLH
6	50	44	73
9	60	60	100
12	80	80	60

Experiment t #	Ab2					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
6	-	-	-	-	-	+
9	-	-	+	-	-	+
12	-	-	+	-	-	+

Experiment t #	Ab3					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
6	-	-	+	+	+	+
9	-	-	-	+	+	+
12	-	-	-	+	+	+

21

25

Experiment t #	Ab3 peptide					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
6	-	-	±	+	+	+
9	-	-	-	-	±	-
12	-	-	-			

Experiment t #	Ab2 + Ab3					
	Before tumor inoculation			After tumor inoculation		
	PBS	control MAb-KLH	AR47.47- KLH	PBS	control MAb-KLH	AR47.47- KLH
6	-	-	-	-	-	+
9	-	-	+	-	-	+
12	-	-	+	-	-	+

13

A lower tumor burden in the group of mice treated with AR 47.47 was observed in 1 experiment out of 3 for Balb/c mice tumor model and in 2 experiments out of 3 for the DBA mice tumor model. The same ELISA assays described in the previous paragraph were used to measure the humoral immune response in mice after tumor inoculation. The presence of Ab2 was detected in the groups of mice immunized with AR 47.47 in all experiments. The presence of Ab3 was detected in all groups of mice in all experiments. The positive signal obtained for Ab3 in the control groups (PBS and control mab) is not surprising since the release of human PSA by the growing tumor in vivo will induce an anti-PSA immune response.

Example 12. EFFECT OF AR 47.47 ON TUMOR PROGRESSION

The ability of AR 47.47 to inhibit the progression of PSA-transfected tumors was studied in both Balb/c mice (experiment # 8, 13) and DBA mice (experiment 10, 14). In

1 this set of experiment the tumor inoculation occurs before treatment of the mice with either
 AR 47.47, control MAb or PBS.

5 For each experiment, the following parameters were measured: Ab2; Ab3; Ab2 +
 Ab3; and tumor burden.

9 The results obtained for each experiment are resumed in the following tables:
 Effect of AR47.47-KLH immunization on Line-1 PSA tumor progression in Balb/c mice

experiment #	# of lung tumor foci (mean ± SE)		
	PBS	control MAb-KLH	AR47.47-KLH
8	14.0 ± 4.6	12.8 ± 2.8	13.0 ± 4.7
13	4.2 ± 1.0	5.0 ± 1.1	7.68 ± 2.2

experiment #	Ab2		
	PBS	control MAb-KLH	AR47.47-KLH
8	-	-	±
13	-	-	-

experiment #	Ab3		
	PBS	control MAb-KLH	AR47.47-KLH
8	+	+	+
13	-	-	-

experiment #	Ab peptide		
	PBS	control MAb-KLH	AR47.47-KLH
8	+	±	±
13	-	-	-

experiment #	Ab2 + Ab3		
	PBS	control MAb-KLH	AR47.47-KLH
8	-	-	+
13	-	-	-

1 Effect of AR47.47-KLH immunization on P815-PSA tumor progression in DBA mice

experiment #	% of mice without tumor		
	PBS	control MAb-KLH	AR47.47-KLH
10	100	100	100
14	100	100	80

experiment #	Ab2		
	PBS	control MAb-KLH	AR47.47-KLH
10	-		+
14	-	-	+

experiment #	Ab3		
	PBS	control MAb-KLH	AR47.47-KLH
10	+	+	+
14	-	+	+

experiment #	Ab peptide		
	PBS	control MAb-KLH	AR47.47-KLH
10	-		-
14	-	-	-

experiment #	Ab2 + Ab3		
	PBS	control MAb-KLH	AR47.47-KLH
10	-	-	+
14	-	-	-

25 No therapeutic effect on tumor progression was observed for AR47.47. However it
should be taken into consideration that the growth rate of tumors in mice far exceed the time
required for the development of an appropriate therapeutic immune response capable to
eradicate or slow tumor progression. Also the growth rate of tumor in mice do not reflect

- 1 what is happening in humans since prostate cancer is considered to be a slow progressing
 disease.

5 **Example 13.** Possible mechanism of action

AR 47.47 Injection (I.V.)		
↓	↓	↓
induction of an idiotypic immune response	interaction with circulating PSA	interaction with PSA at the tumor site
↓	↓	↓
Ab2/Ab3	processing of tumor antigen by APC	migration of immune cells at the tumor site
↓	↓	↓
PSA specific CTLs	PSA specific CTLs	induction of an immune response against various tumor antigens
↓	↓	↓

17

Although the present invention has been described in terms of a particular preferred embodiments, it is not limited to those embodiments. Alternative embodiments, examples, and modifications which would still be encompassed by the invention may be made by those skilled in the art, particularly in light of the foregoing teachings.

1 We claim:

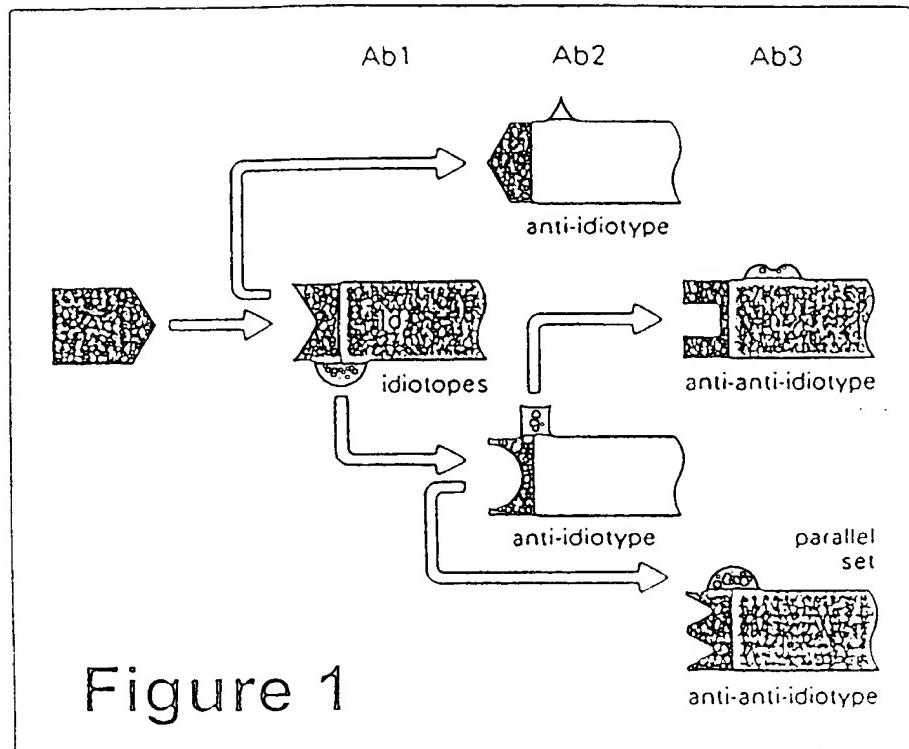
1. A composition for the treatment of prostate cancer comprising a composition comprising a binding agent that specifically binds to circulating prostate specific antigen and induces a therapeutic immune response.
- 5 2. A method for treating prostate cancer comprising administering an effective amount of a composition comprising a binding agent that specifically binds to circulating prostate specific antigen and induces a therapeutic immune response.
- 9 3. The composition of claims 1 or 2 wherein the binding agent binds to an epitope of prostate specific antigen comprising amino acid sequences 139 to 163.
- 13 4. The composition wherein the composition comprises a binding agent that has ATCC Accession No. 12526.
- 17 5. The composition and method of claims 1 and 2 wherein the therapeutic immune response comprises a humoral and cellular immune response.
- 21 6. A composition for treating prostate cancer comprising a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.
- 25 7. A composition for inducing an immune response comprising a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.
- 29 8. A composition for increasing the immunogenicity of an antigen comprising a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.

- 1 9. A composition for the treatment of prostate cancer wherein the composition comprises a binding agent that binds to the same epitope as the epitope bound by an antibody produced by the hybridoma that has ATCC Accession No. 12526.
- 5 10. The compositions of claims 1-9 wherein the binding agent is conjugated to an immunogenic carrier.
- 9 11. The compositions of claim 10 wherein the immunogenic carrier is keyhole limpet hemocyanin.
- 13 12. A method for treating prostate cancer comprising administering a composition comprising a binding agent that specifically binds to circulating prostate specific antigen and inducing a therapeutic immune response to the antigen.
- 17 13. The method of claims 2 or 12 wherein the binding agent binds to an epitope of prostate specific antigen comprising amino acid sequences 139 to 163.
- 21 14. The method of claims 12 or 13 wherein the composition comprises a binding agent that has ATCC Accession No. 12526.
- 25 15. The method of claims 12 to 14 wherein the therapeutic immune response comprises a humoral and cellular immune response.
- 29 16. A method for treating prostate cancer comprising administering a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.
17. A method for inducing an immune response comprising administering a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.

1

18. A method for increasing the immunogenicity of an antigen comprising administering a binding agent that specifically binds to an epitope of circulating prostate specific antigen, the binding agent being capable of binding to the antigen to form an immunogenic binding agent-antigen complex.
- 5
19. A method for the treatment of prostate cancer wherein the composition comprises a binding agent that binds to the same epitope as the epitope bound by an antibody produced by the hybridoma that has ATCC Accession No. 12526.
- 9
20. The methods of claims 12-19 wherein the binding agent is conjugated to an immunogenic carrier.
- 13
21. The methods of claim 20 wherein the immunogenic carrier is keyhole limpet hemocyanin.

17



E	E									
139	140									
F	L	T	P	K	K	L	Q	C	V	
141	142	143	144	145	146	147	148	149	150	
D	L	H	V	I	S	N	D	V	C	
151	152	153	154	155	156	157	158	159	160	
A	Q	V								
161	162	163								

Figure 2

10

(Seq ID No.: 1)

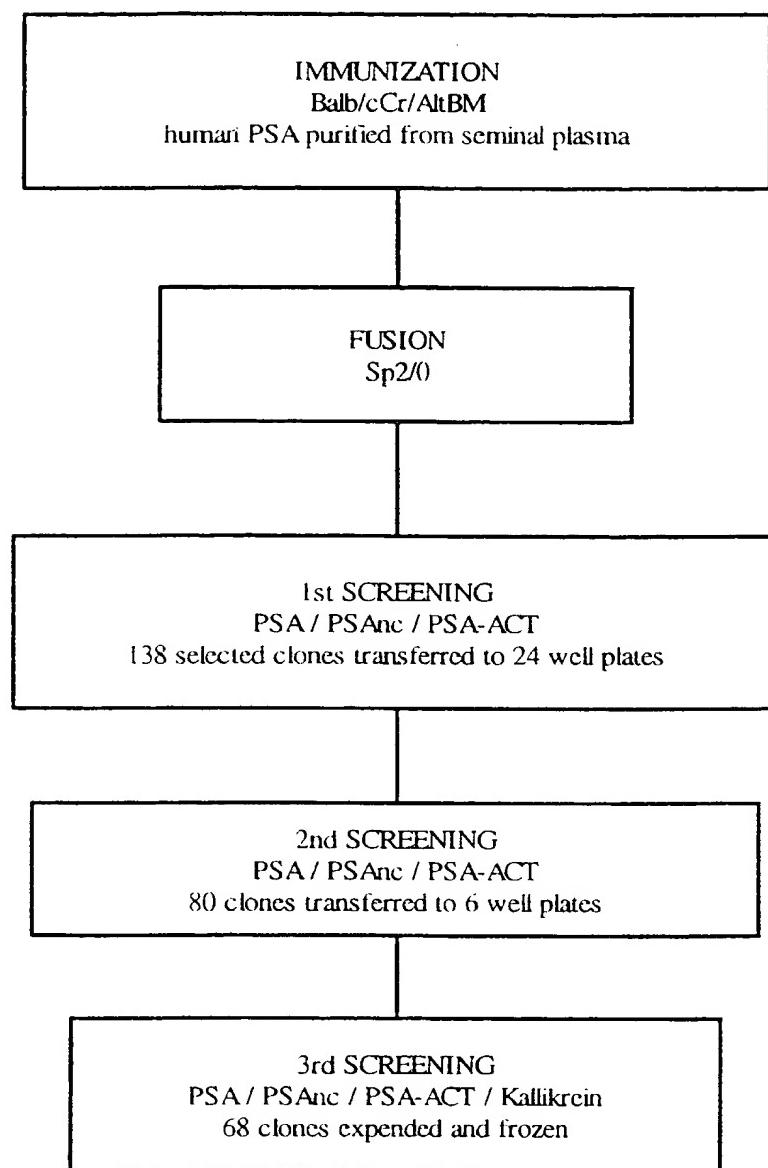


Figure 3

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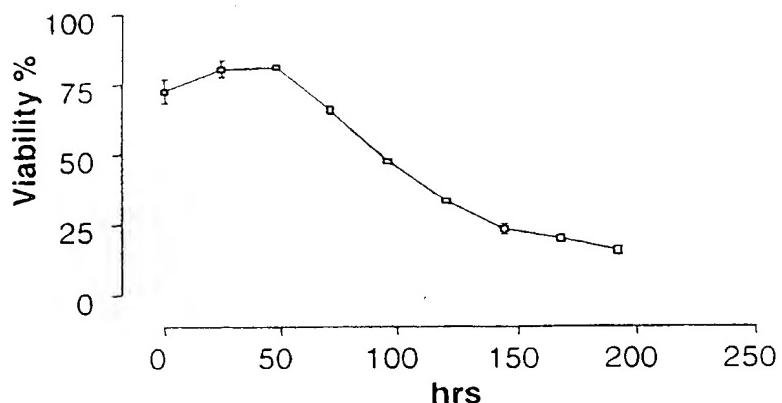


Figure 4a

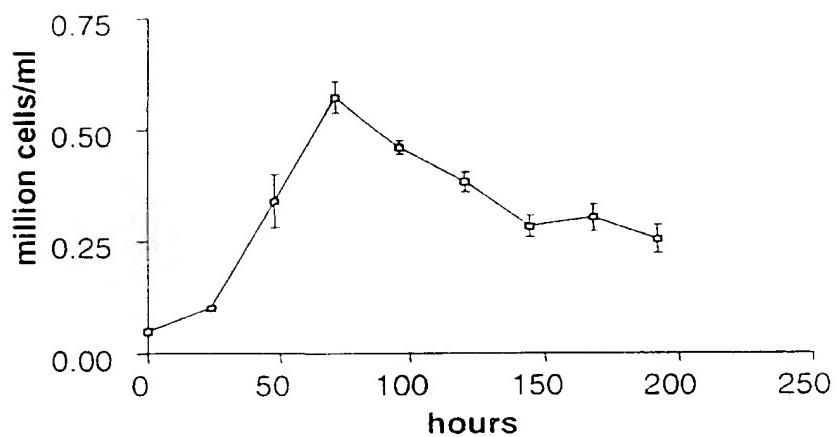


Figure 4b

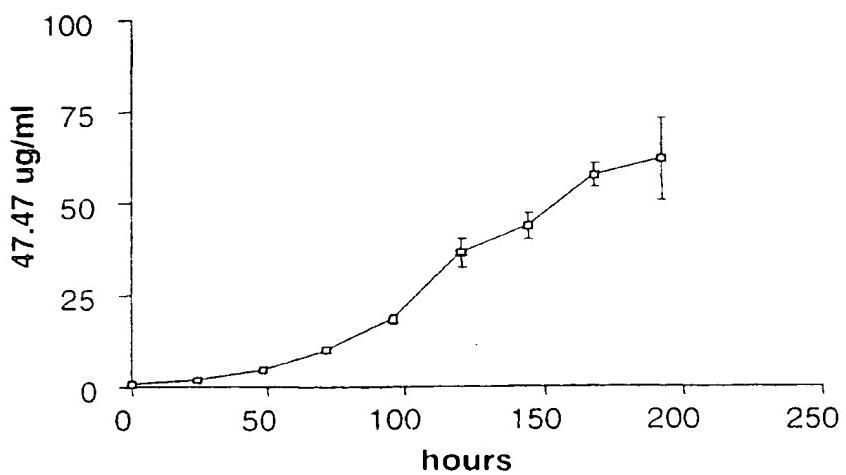
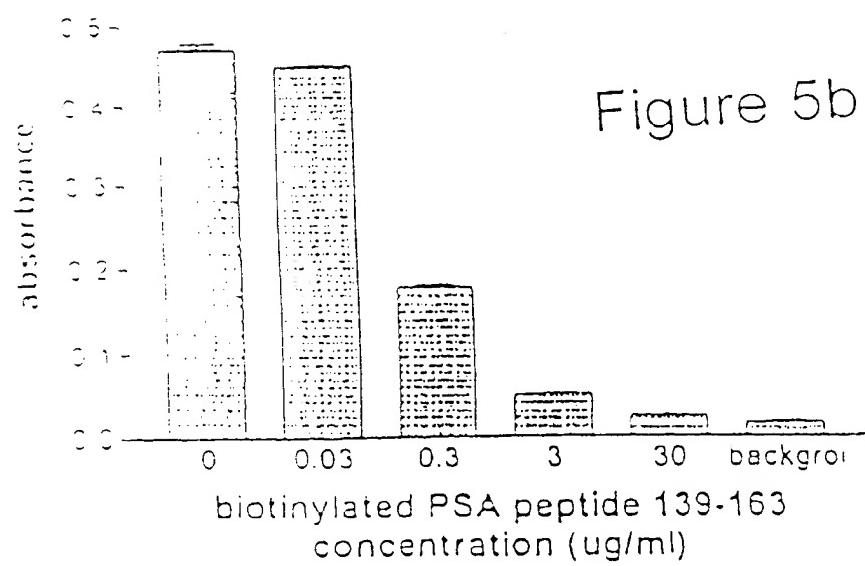
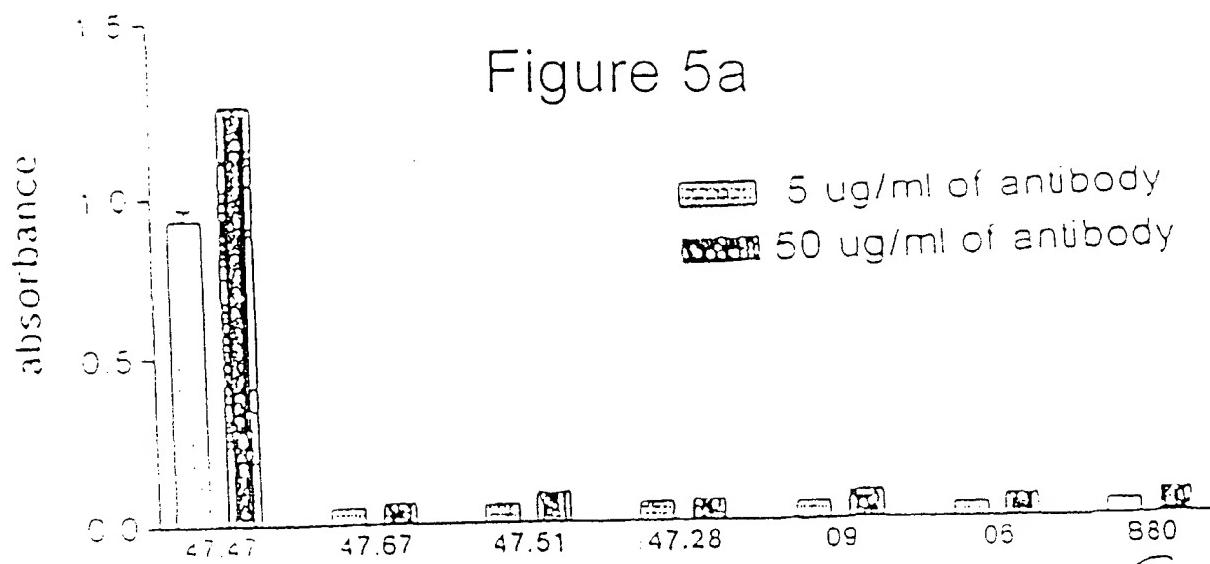


Figure 4c



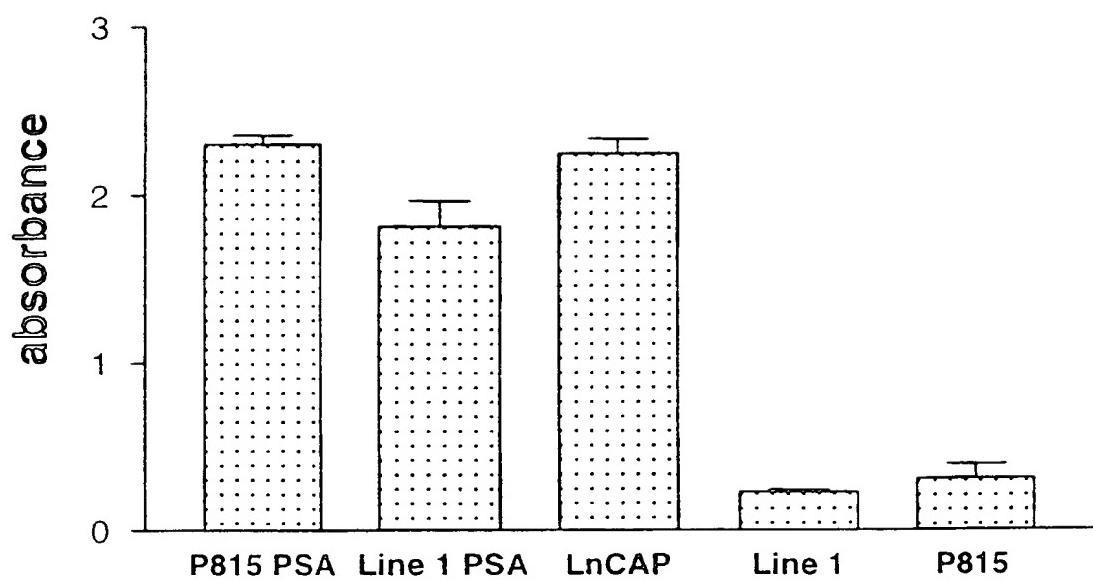


Figure 6

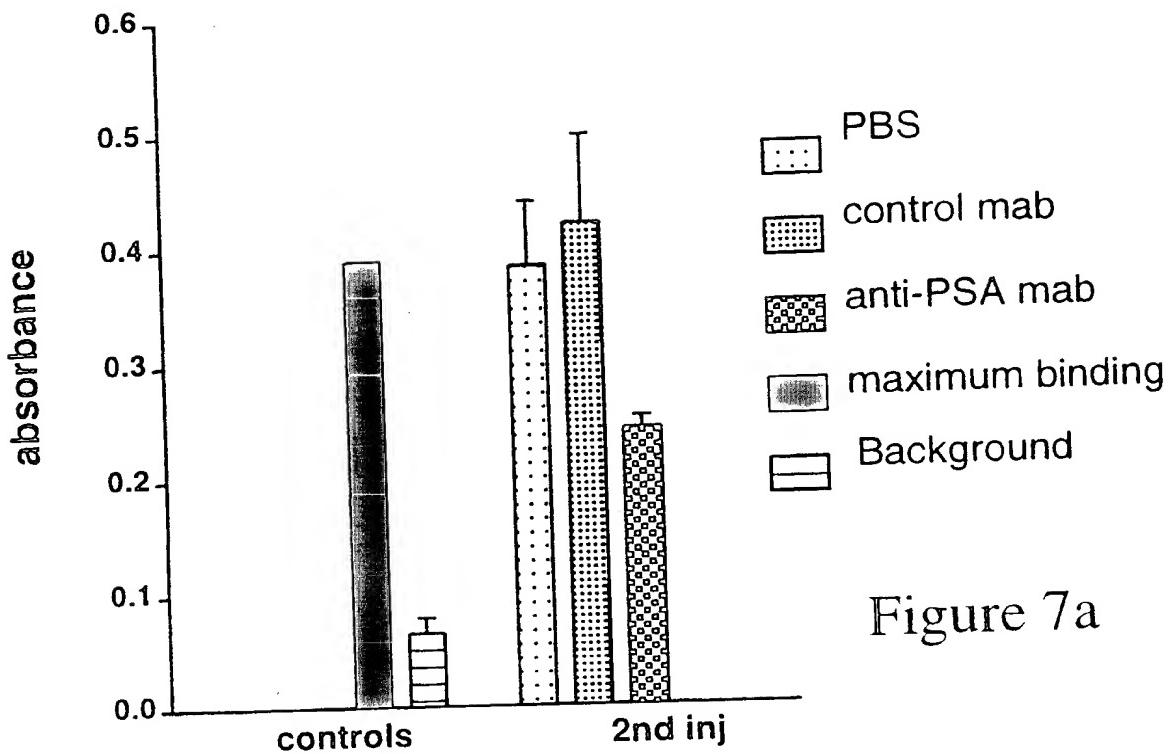


Figure 7a

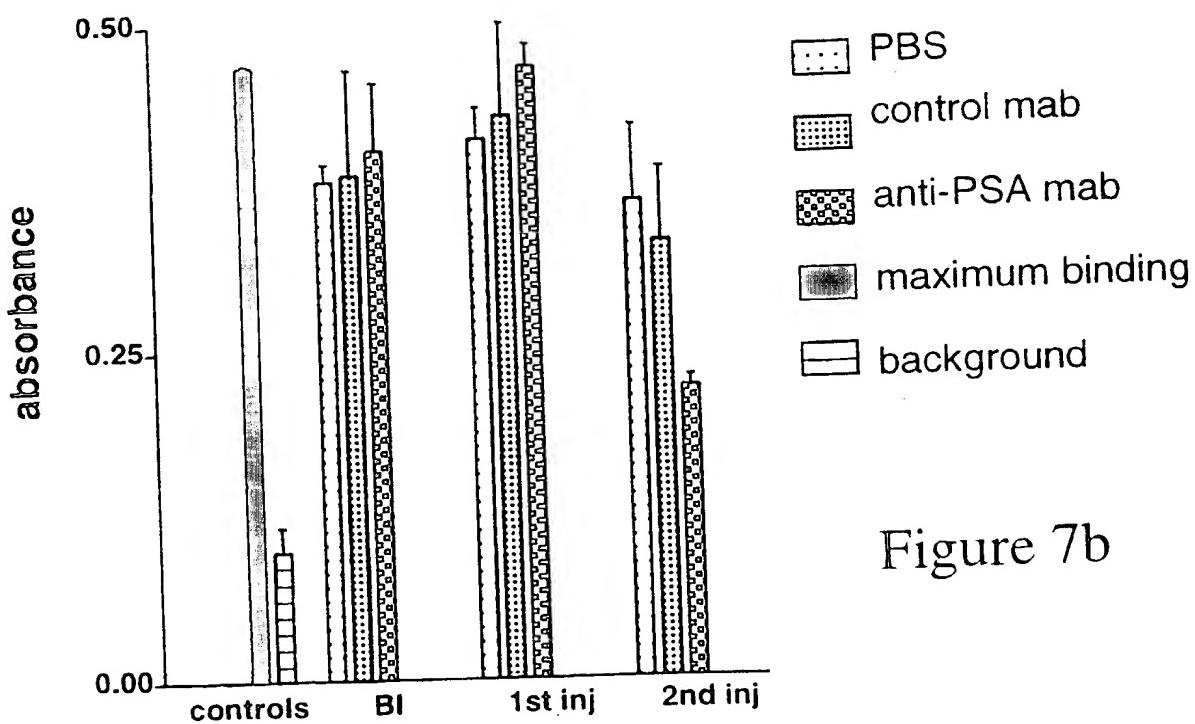
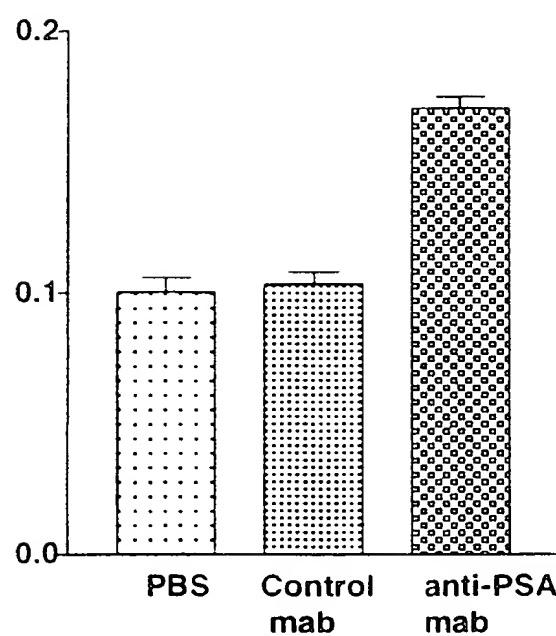
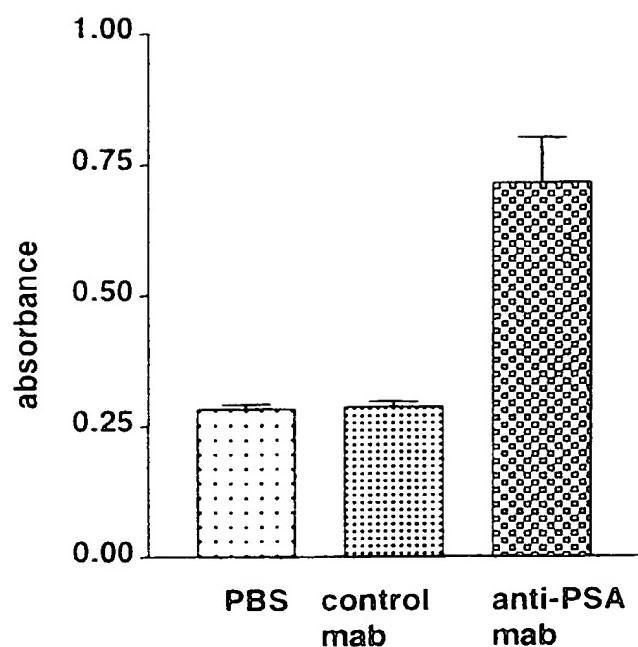


Figure 7b



Balb/c mice

Figure 8a



DBA mice

Figure 8b

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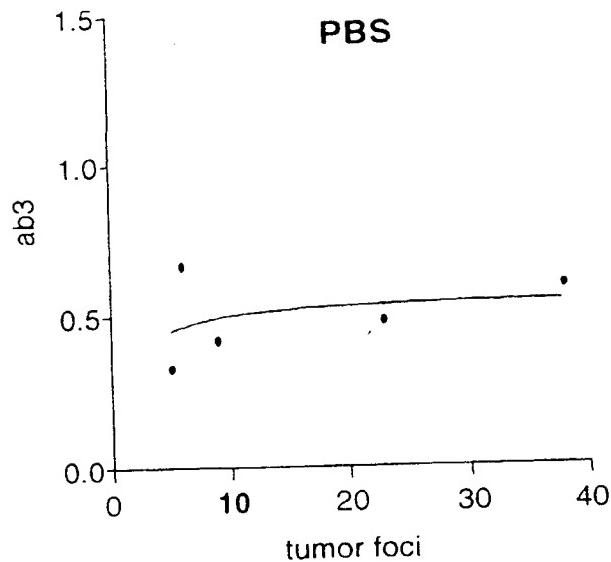


Figure 9a

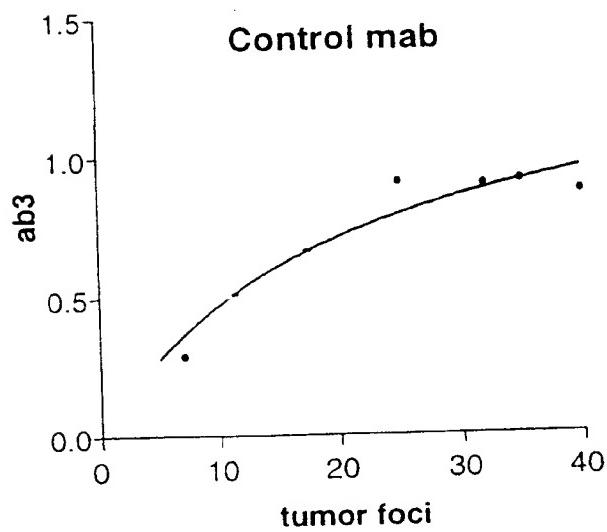


Figure 9b

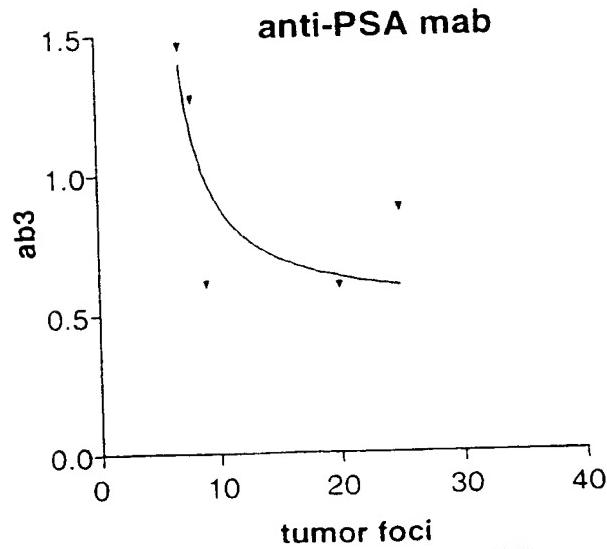


Figure 9c

Figure 10a

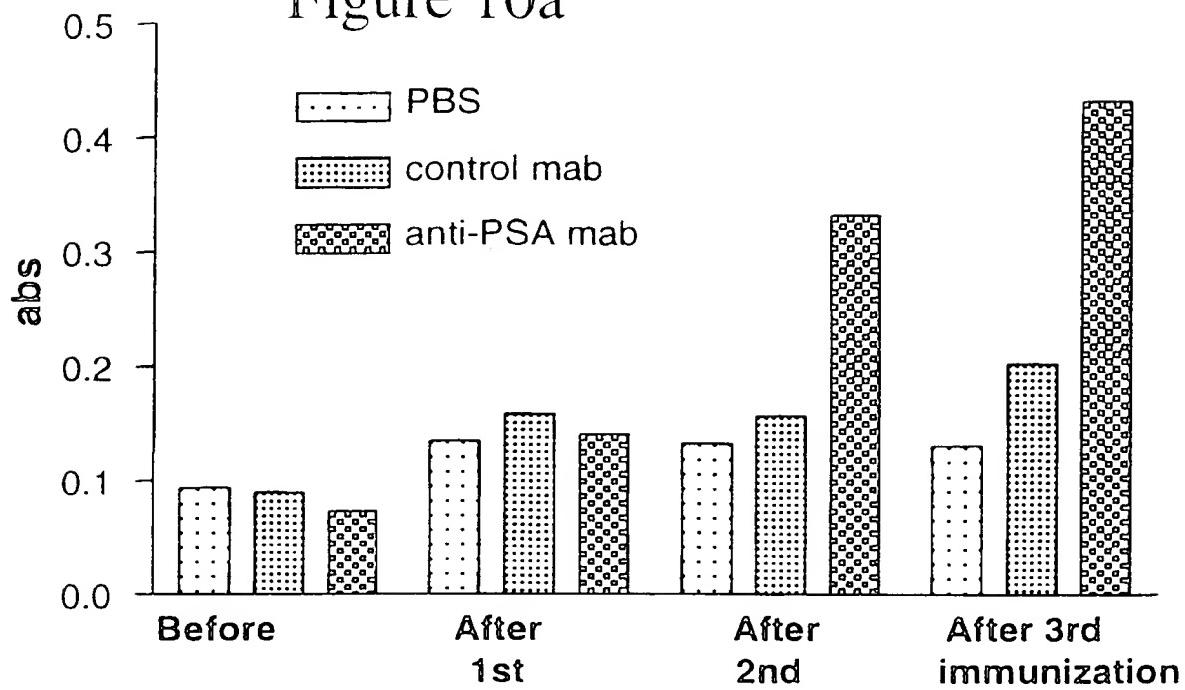
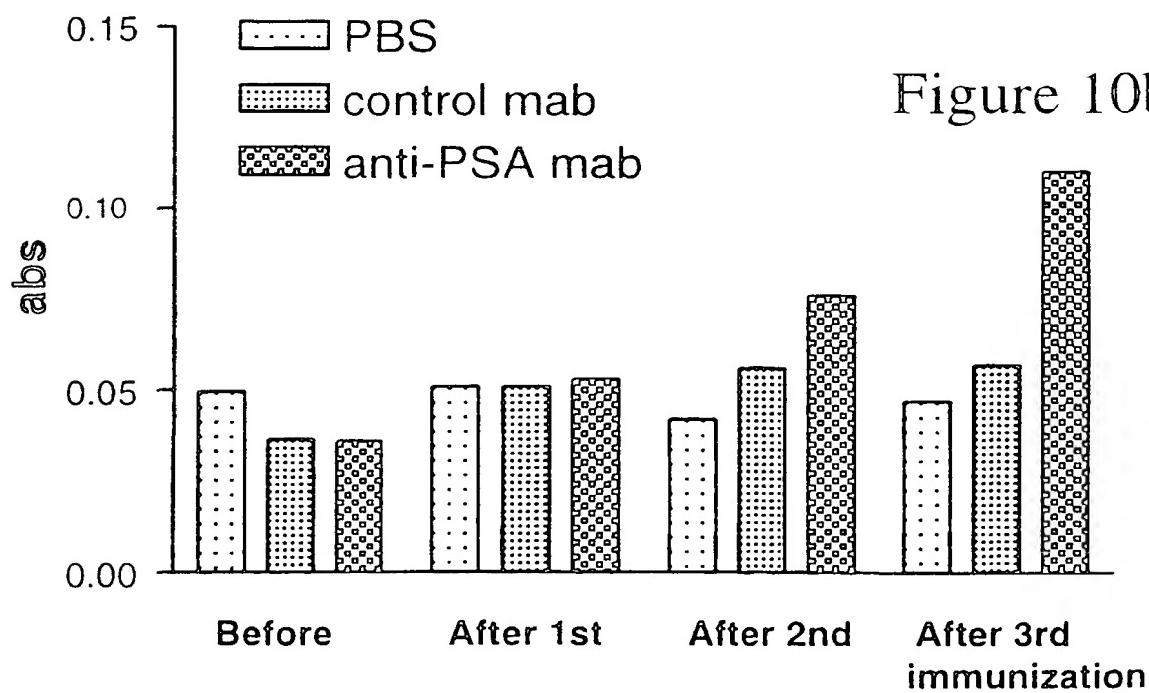


Figure 10b



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INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01115

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K39/395 A61K39/385 //C07K16/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	UEMURA H ET AL: "Generation of anti-idiotype antibodies related to PSA: possible tools for treatment of prostate cancer." JOURNAL OF UROLOGY, vol. 153, no. SUPPL 4, 1995, page 380A XP002117650 abstract	1,2,5-8, 10-12, 15-18, 20,21
Y	---	3,4,9, 13,14,19



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority, claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

6 October 1999

18/10/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Le Flao, K

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/IB 99/01115

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CORREALE P ET AL: "In vitro generation of human cytotoxic T lymphocytes specific for peptides derived from prostate-specific antigen." JOURNAL OF THE NATIONAL CANCER INSTITUTE, vol. 89, no. 4, 19 February 1997 (1997-02-19), pages 293-300, XP002039189 abstract ---	3,4,9, 13,14,19
X	LEVEUGLE B ET AL: "PSA-directed immunotherapy of prostate cancer." PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 39, March 1998 (1998-03), page 355 XP002117652 abstract ---	1,2,5-8, 10-12, 15-18, 20,21
A	WO 97 29199 A (ABBOTT LABORATORIES) 14 August 1997 (1997-08-14) claims 1-8 ---	1-21
A	EP 0 652 014 A (NATIONAL INSTITUTE OF IMMUNOLOGY) 10 May 1995 (1995-05-10) claims 1-10; examples 3-6 ---	1-21
A	WO 98 10292 A (CENTOCOR) 12 March 1998 (1998-03-12) claim 10 -----	1-3,12, 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 99/01115

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 2 and 12-21 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 99/01115

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9729199 A	14-08-1997	EP 0879290 A	25-11-1998
EP 0652014 A	10-05-1995	NONE	
WO 9810292 A	12-03-1998	AU 4086397 A	26-03-1998

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